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Comparison of three real-time PCR methods with blood smears and rapid diagnostic test in *Plasmodium* sp. infection

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Abstract

In cases of malaria, rapid and accurate diagnosis of *Plasmodium* sp. is essential. In this study three different quantitative, real-time PCR methods were compared with routine methods used for malaria diagnosis. A comparative study was conducted prospectively in the laboratories of Montpellier and Nîmes University Hospitals. The methods used for routine diagnostic malaria testing consisted of microscopic examination of Giemsa-stained blood smears and rapid diagnostic tests. Three quantitative real-time PCR methods (qRT-PCR) were tested: qRT-PCR1 amplified a specific sequence on the *P. falciparum* Cox1 gene, qRT-PCR2 amplified a species-specific region of the multicopy 18S rDNA, and qRT-PCR3 amplified a mitochondrial DNA sequence. Among the 196 blood samples collected, 73 samples were positive in at least one of the five tests. Compared with the routine method, there were no false negatives for *P. falciparum* diagnosis in either qRT-PCR1 or qRT-PCR3. In all *P. ovale*, *P. vivax* and *P. malariae* infections diagnosed from blood smears, qRT-PCR1 was negative, as expected, whereas qRT-PCR2 and qRT-PCR3 were positive and concordant (simple κ coefficient = 1). One negative sample from microscopy was positive with both qRT-PCR2 and qRT-PCR3. Together, qRT-PCR3 and the combined qRT-PCR1 and qRT-PCR2 were concordant with routine methods for malaria diagnosis (99% and 99.5%, respectively). These three rapid, molecular qRT-PCR methods, used alone or in association, showed excellent results, with high concordance, accuracy and reliability in malaria diagnosis.

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Introduction

In tropical areas, malaria primarily caused by *Plasmodium falciparum* [1] remains a leading cause of morbidity and mortality. In metropolitan France, almost all diagnosed cases of malaria are imported, with the exception of a few particular forms (malaria transmission in airports and via blood transfusion). In all cases, rapid diagnosis with species identification is essential to optimize treatment. The microscopic examination of blood smears has been considered the reference standard method for diagnosis. For several years, alternative diagnostic methods such as rapid diagnostic tests (RDTs) have been available [2,3]. Most of these rely on immunochromatography RDTs to detect histidine-rich protein 2 (HRP2), specific to *P. falciparum*, and *Plasmodium* lactate dehydrogenase or aldolase, enzymes common to all species. In cases of very low parasitaemia or if self-medication has taken place before medical consultation, results of microscopic examination can be negative while RDT results remain positive. However, rare false-positive detection has been reported, due to cross-reaction with rheumatoid factors, and a lack of sensitivity of RDT has been reported especially for *P. ovale*, *P. vivax* and *P. malariae* [4]. Since the early 1990s, various PCR methods have been tested for detecting *Plasmodium* spp. DNA in blood samples and to identifying species, both in endemic areas and in countries with imported malaria [5–7]. Development of real-time PCR (qRT-PCR) has made possible the use of molecular methods for the emergency diagnosis of malaria. Since the first study published in 2001 [8], many assays have been

TABLE 1. Main characteristics of real-time PCR assays for the diagnosis of malaria

Authors (year)	Study place	Gene target	Detected species	Real-time PCR: Method	Real-time PCR: Sensitivity (parasite/μL)	Real-time PCR: Samples number (positive samples)	Other methods[a]: positive samples
Studies carried out in non-endemic area (imported malaria)							
Lee *et al.* (2002) [9]	Singapore	SSUrRNA	Four sp.[b]	TaqMan probes	Pf: 0.1	153 (127)	OM: 125
Fabre *et al.* (2004) [10]	France	SSUrRNA/Cox1	Genus and four sp.	SybrGreen	Pf: 0.035	183 (69)	OM + RDT: 57 Conventional PCR: 69
Rougemont *et al.* (2004) [11]	Switzerland	SSUrRNA	Genus and four sp.	TaqMan probes (for screening and identification)	0.2–2	66 (37)	OM: 31 Combining OM + RDT + nested PCR: 38
Farcas *et al.* (2004) [12]	Canada	SSUrRNA	Genus	Kit Real Art Malaria LC® (Artus GmbH, Hamburg Germany)	ND	259 (208)	Nested PCR: 209
Perandin *et al.* (2004) [13]	Italy	SSUrRNA	Pf, Pv, Po	TaqMan probes	Pf: 0.7, Po: 4 Pv: 1.5	122 (60)	OM: 61 Nested PCR: 60 + 2 Pm
Elsayed *et al.* (2006) [14]	Canada	Cox1/SSUrRNA	Genus and Pf	Molecular beacon probes	Pf: 0.004 Genus: 0.16	78 (27)	OM: 27 Conventional PCR: 27
Mens *et al.* (2006) [15]	Netherlands	SSUrRNA	Four sp.	Real time QT-NASBA[c]	0.002	79 (77)	OM: 79
Bialasiewicz *et al.* (2007) [16]	Turkey & Australia	SSUrRNA cf. (i) Perandin *et al.* (2004) [13] & (ii) Rougemont *et al.* (2004) [11]	Pf, Pv, Po	TaqMan probes	ND	119 (i) (105) (ii) (106)	No other method
Safeukui *et al.* (2008) [17]	France	SSUrRNA	Four sp.	FRET hybridization probes	Pf: 0.025	119 (93, including 75 Pf)	OM: 86 Conventional PCR 'Genus': 91 Conventional PCR Pf: 71
Studies carried out in endemic area							
Malhotra *et al.* (2005) [18]	Kenya	SSUrRNA	Pf	TaqMan probe	ND	2139 (738/2009)	OM: 265/2139 RDT[d]: 237/941, 72/283 Nested PCR: 1070/1939
Swan *et al.* (2005) [19]	Thailand	SSUrRNA	Genus and four sp.	FRET hybridization probes	10 targets/μL	297 (284)	OM: 292
Mens *et al.* (2007) [20]	Kenya, Tanzania	SSUrRNA	Genus	Real-time QT-NASBA	ND	338 (91)	OM: 63 RDT[d]: 64/338, 62/338, 69/184 Nested PCR: 94
Boonma *et al.* (2007) [21]	Western Thailand	SSUrRNA cf. Mangold *et al.* [24]	Four sp.	SyberGreen	ND	136 (91)	OM: 89 Conventional PCR: 91 Nested PCR: 91
Gama *et al.* (2007) [22]	Brazil	SSUrRNA	Four sp.	TaqMan probe	0.5	64 (33)	OM: 33 RDT: 20/28 Conventional PCR: 33
Veron *et al.* (2008) [23]	French Guiana	SSUrRN	Pf, Pv, Pm	TaqMan probe	Pf: 3.1, Pv: 0.3, Pm: 0.8	263 (189)	OM: 188 Nested PCR: 189
Studies carried out in endemic and non-endemic areas							
Mangold *et al.* (2005) [24]	USA, Malaysia, Myanmar, Thailand	SSUrRNA	Four sp.	SybrGreen	1	358 (74)	OM: 76
Vo *et al.* (2007) [25]	Europe (E) Vietnam (V)	AQP (Pf), ECPR (Pv), POS25 (Po), CS (Pm)	Four sp.	SybrGreen	Pf: 15; Pv: 10 Po, Pm: 25	(E) 56 (47) (V) 134 (69)	OM: 44 OM: 60

ND, not determined.
[a]Methods including: optical microscopy (OM); rapid diagnostic test (RDT) and conventional and nested PCR methods.
[b]Pf, *Plasmodium falciparum*; Po, *P. ovale*; Pv, *P. vivax*; Pm, *P. malariae*.
[c]Quantitative nucleic acid sequence-based amplification.
[d]Results obtained according to the rapid diagnostic test used.

developed (Table 1) [9–25]. Nevertheless, results should be interpreted whether the method was used for diagnosis in patients with clinical signs of disease or for epidemiological studies in endemic areas.

The main purpose of this study was to assess the accuracy of three molecular methods for the diagnosis of imported malaria in hospital laboratories. The presence of *Plasmodium* spp. parasites in blood samples was assessed

by thin blood smear microscopic examination associated with a RDT and three qRT-PCR methods. One PCR method detected only *P. falciparum* (qRT-PCR1), another method (qRT-PCR3) amplified all four species DNA but could only distinguish *P. falciparum* from non-*P. falciparum* (*P. vivax*, *P. ovale* and *P. malariae*), and the last method (qRT-PCR2) amplified and identified the four species.

Material and Methods

Patients and collection of samples

A prospective study was conducted between August 2005 and 2007 in parasitological laboratories of both Montpellier and Nîmes University Hospitals. One hundred and ninety-two patients were included in this study. A total of 196 blood samples were sent to the laboratories for routine diagnosis of malaria. The results obtained with three qRT-PCRs were compared with those of the methods used routinely. The study was approved by the ethics committee of Nîmes University hospital.

Routine methods

Blood samples were collected in EDTA-containing tubes for routine and qRT-PCR methods. In all cases, biological diagnosis was based on microscopic examination of thin blood smears associated with RDT. The Now® Malaria test, (Binax, Scarborough, ME, USA), detecting HRP2 antigen and aldolase from 15 μL of blood, was used according to the manufacturer's instructions. For each sample, four thin blood smears (corresponding to a total of at least 20 μL of blood) were examined by two well-trained microscopists for at least 20 min (eyepiece ×10, objective ×50 and ×100) before a negative result was concluded.

DNA extraction

DNA was extracted from 200 μL of whole blood with QIAamp® DNA Mini kit Qiagen (Courtaboeuf, France), for the 181 first samples and with Biorobot EZ1 Qiagen® automat and EZ1 DNA Blood 200 μL kit Qiagen® for the 15 last samples, according to the manufacturer's recommendations. The DNA samples were eluted in a final volume of 200 μL and stored at −20°C.

Real-time PCR

SybrGreen (Fast Start DNA Master SybrGreen I™, Roche Diagnostics, Meylan, France) was used for detection in a LightCycler™ instrument (Roche Diagnostics, Meylan, France). The primers for qRT-PCR1 (5′-TTACATCAGGAATGTTATTGC-3′ and 5′-ATATTGGATCTCCTGCAAAT-3′) [14] were used to amplify a 120-bp sequence of the *P. falciparum* mitochondrial cytochrome c oxidase (Cox1) gene.

The primers for qRT-PCR2 (5′-TAACGAACGAGATCTTAA-3′ and 5′-GTTCCTCTAAGAAGCTTT-3′) [24] were used to amplify a species-specific region of the multicopy 18S rRNA gene. Indeed, the 18S rDNA contains sequences that are highly conserved across *Plasmodium* spp. (genus-specific regions), interspersed with species-specific regions. The primers for the third, qRT-PCR3, (5′-TAGCCGACAAGGAATTTTGC-3′ and 5′-CCTTGAATGGAGCACTGGAT-3′) were designed using Primer 3 software to amplify a mitochondrial sequence (191–193 bp) common to all *Plasmodium* spp. All acquired fluorescence data were analysed using LightCycler software. Melting curve analysis was used for characterization of the qRT-PCR product and for species differentiation depending on the method of PCR.

Average melting curve peaks Tm (± standard deviation) for *Plasmodium* sp. DNA controls were 73.28 ± 0.28°C for *P. malariae*, 75.38 ± 0.16°C for *P. falciparum*, 77.17 ± 0.13°C for *P. ovale* and 78.74 ± 0.31°C for *P. vivax*.

Primer specificity was first checked using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi), then tested with human, bacterial (*Salmonella* sp. and *Escherichia coli*), viral [pro-viral human immunodeficiency (HIV)-1 DNA, cytomegalovirus, HIV-1 RNA] and parasital DNA (*Leishmania* sp. and *Trypanosoma* sp.). DNA extraction and the absence of PCR's inhibitory substances were tested by Tissular Plasminogen Activator gene amplification in all the samples. Each method of qRT-PCR was optimized using DNA of the four *Plasmodium* species. Hybridization temperatures retained were 56, 54 and 58°C for qRT-PCR1, -2 and -3, respectively. Each 20-μL reaction capillary contained 5 μL of DNA and 15 μL of PCR reaction mix. The intra- and inter-test reproducibility of Tm and Ct were evaluated with 14 *Plasmodium* spp. DNA control samples by testing the same control sample three times in the same run, in ten different runs. Efficacy was calculated with four dilutions of *P. falciparum*, *P. ovale*, *P. vivax* and *P. malariae* DNA control samples for qRT-PCR2 and -3. Efficacy and absolute sensitivity were estimated for qRT-PCR1 with a plasmid construction. Standards of cloned template (2×10^4, 2×10^3, 2×10^2, and 20 copies/μL) were tested to generate a standard curve. During the entire study, one to four DNA control samples of *P. falciparum*, *P. ovale*, *P. vivax* or *P. malariae*, depending on the qRT-PCR method, were tested in each assay.

Cloned template

The Cox-1 single copy gene target sequence was cloned into the 'pDrive Cloning Vector' using the Qiagen PCR

Cloning^{plus} kit® (Roche Diagnostics, Meylan, France). After purification of the plasmid, the concentration of the template (42.5×10^6 copies/μL) was derived from the optical density measurement (84 ng/μL) and the plasmid molecular weight. Tenfold serial dilutions of the plasmid were prepared (2×10^4, 2×10^3, 2×10^2, 20 and two copies per tube).

Statistical analysis

The different qRT-PCR methods were compared with each other and with the routine methods. Kappa coefficient and the McNemar test were performed. To insure that concordance was not due to hazard effect, the κ coefficient was tested to 0 (i.e. κ coefficient representative of hazard effect). Concordance between methods was calculated for infections involving all species and then separately for *P. falciparum* infection alone and for 'non-*P. falciparum*' (*P. ovale*, *P. vivax* and *P. malariae*) infections. Analysis was conducted using the SAS program, version 8 (SAS Institute Inc., Cary, NC, USA). All p values ≤ 0.05 were considered statistically significant.

Results

Population characteristics

The population comprised 192 travellers, returned from malaria-endemic areas or migrants recently arrived in France. All patients presented at the hospitals with fever or recent history of fever. All blood samples were collected during consultation with a physician in one of the two hospitals. Malaria was diagnosed for 72 (37.5%) patients by microscopy and/or RDT. Six had cerebral malaria. The majority (84.5%) of total cases were known to have been acquired in Africa, especially on the Ivory Coast. *Plasmodium falciparum* was the main species (81.9%). Malaria caused by *P. falciparum* occurred within a mean of 15 days (±10 days) after returning from an endemic area and usually longer (33–318 days) for *P. vivax*, *P. ovale* or *P. malariae*.

qRT-PCR performances

To determine qRT-PCR performances, after optimization, specificity, reproducibility, efficacy and absolute sensitivity were evaluated only for qRT-PCR1. qRT-PCR1 allowed specific diagnosis of *P. falciparum* only, as expected, and did not amplify *P. ovale*, *P. vivax* or *P. malariae* DNA, whereas qRT-PCR2 and -3 amplified all four species. No amplification was obtained from samples of non-*Plasmodium* DNAs used to test the specificity of the three qRT-PCR methods. For qRT-PCR2, melting curve analysis was performed to determine the melting temperature allowing species identification. Patient specimens' average melting curve peaks Tm were 73.46 ± 0.21°C for *P. malariae*, 75.33 ± 0.34°C for *P. falciparum*, 77.29 ± 0.44°C for *P. ovale* and 79.55 ± 0.47°C for *P. vivax*. qRT-PCR3 melting curve analysis leads to *P. falciparum* or 'non-*P. falciparum*' diagnosis. The melting temperature was exactly the same for *P. ovale*, *P. vivax* and *P. malariae*. One degree separated *P. falciparum* melting curve peaks Tm from the others. The three qRT-PCR methods showed excellent reproducibility and efficacy (Table 2). As regards qRT-PCR1, absolute sensitivity was estimated to <1 parasite/μL of whole blood.

Blood sample results

For the 192 patients suspected of having malaria, 196 samples were sent to the laboratories. The results of the three qRT-PCRs are summarized in Table 3. Overall, 73 samples were found positive by at least one qRT-PCR method, 59 positive for *P. falciparum*, eight for *P. ovale*, four for *P. vivax* and two for *P. malariae*. Regarding *P. falciparum* infections, 89.8% (53/59) were positive with all routine and molecular methods. In all the six other cases (6/59), qRT-PCR3 was positive. RDT and qRT-PCR1 were positive in 5/6 cases,

TABLE 2. Reproducibility and efficacy of qRT-PCR methods

	P. falciparum	*P. vivax*	*P. ovale*	*P. malariae*
Ct and Tm reproducibility				
Ct and Tm CV for qRT-PCR1	0.004–0.0044	/	/	/
Ct and Tm CV for qRT-PCR2	0.02–0.0037	0.023–0.0029	0.023–0.0032	0.019–0.0032
Ct and Tm CV for qRT-PCR3	0.031–0.0027	0.002–0.0018	0.005–0.002	0.001–0.002
Efficacy				
qRT-PCR1	1.9	/	/	/
qRT-PCR2	1.87	2	1.94	1.99
qRT-PCR3	1.96	1.95	1.97	1.91

Reproducibility was based on the melting curve peaks, Tm analysis, and on inter-assay threshold cycle (Ct) analysis. qRT-PCR intra- and inter-test reproducibility was evaluated with 14 *Plasmodium* sp. DNA control samples, by testing the same control sample in triplicate in the same run, and ten different runs. Mean, standard deviation and the coefficient of variation were calculated.
Efficacy was calculated with four dilutions of *P. falciparum*, *P. ovale*, *P. vivax* and *P. malariae* DNA control samples for qRT-PCR2 and -3, and with a plasmid construction for qRT-PCR1.
CV, coefficient of variation.

TABLE 3. Results of the different diagnostic methods in patients with clinical suspicion of malaria

Results	Methods					
	Microscopy	Rapid diagnostic test[a]	qRT-PCR1[b]	qRT-PCR2[c]	qRT-PCR3[d]	qRT-PCR1 + -2[e]
Negative	130	128	137	126	116	123
Non-contributory[f]	0	0	1	1	7	1
P. falciparum	53	58	58	55	59	58
P. vivax	4	4	0	4	4	4
P. ovale	7	5	0	8	8	8
P. malariae	2	1	0	2	2	2
Total	66	68	58	69	73	72

[a]Detects HRP2 antigen and aldolase.
[b]Detects only *P. falciparum* infection.
[c]Detects and distinguishes the four *Plasmodium* species.
[d]Detects the four species and distinguishes *P. falciparum* from the three other species.
[e]Combines results of qRT-PCR1 and -2.
[f]These results do not permit either a positive or a negative conclusion but require another method of diagnosis.

whereas qRT-PCR2 was only positive in two cases. Blood smear examination was negative in all these six cases. Thus, qRT-PCR3 detected only one additional *P. falciparum* infection. This patient was not treated and the case was considered as a spontaneous healing. Indeed, this migrant patient had come from Senegal 8 weeks before and presented a fever that disappeared without treatment. Moreover, during the clinical follow-up (after 8 weeks), no other malaria symptoms were observed. Contamination of the qRT-PCR assay was excluded from the negative results in the negative controls and by the positive results obtained on two new runs. Regarding *P. ovale*, *P. vivax* and *P. malariae* infections, comparing microscopy and RDT with qRT-PCR2 and -3, we found one and four false-negative samples in blood smear examination and in RDT, respectively. RDT sensitivity was only 71% (10/14). One *P. ovale*-positive sample was detected only by qRT-PCR2 and -3. Actually, this *P. ovale* infection was confirmed by blood smear examination 2 days later with a second sample sent to the laboratory for *Plasmodium* sp. detection. Either qRT-PCR2 or -3 would have allowed early diagnosis of this infection. For all positive samples, species discrimination was fully concordant with blood smear examination. As expected, qRT-PCR1 did not show any DNA amplification for 'non-*P. falciparum*' species. There were no false-positive results for RDT during the entire study. Moreover, whatever the method of *Plasmodium* sp. diagnosis used, no mixed infection was ever detected. Concerning the non-contributory results recorded for molecular methods (Table 3), a slight and late amplification on SybrGreen with an appropriate Tm was observed. A non-contributory result can not be interpreted either as positive or negative. In this case, another diagnostic method is required. In each case, the four other methods were negative and clinical evolution confirmed the absence of malaria infection.

Concordance rates

The three qRT-PCR methods were compared with microscopic examination of blood smear combined with RDT (Table 4). qRT-PCR1 was fully concordant with routine methods in *P. falciparum* infections. The concordance rate with routine methods was 99.5% for the association of qRT-PCR1 and -2. Except for the comparison between optical

TABLE 4. Comparison of routine and molecular diagnostic methods for suspected malaria

	OM[a] + RDT[b] vs. qRT-PCR1[c]	OM + RDT vs. qRT-PCR2	OM + RDT vs. qRT-PCR3	OM + RDT vs. qRT-PCR1 + -2
P. falciparum infections (*n* = 59)				
Concordant rate (%)	100	95	98.3	100
Kappa coefficient (95% CI)	1	0.38 (0–0.92)*	NC	1
P. ovale, *P. vivax*, *P. malariae* infections (*n* = 14)				
Concordant rate (%)	7[c]	92.9	92.9	92.9
Total samples (*n* = 196)				
Concordant rate (%)	93.3[c]	98	99	99.5
Kappa coefficient (95% CI)	0.85 (0.77–0.93)**	0.96 (0.91–1)**	0.98 (0.95–1)**	0.99 (0.97–1)**
MacNemar test	0.0003	0.32	0.16	0.32

NC, not calculable.
[a]Optical microscopy.
[b]Rapid diagnostic test.
[c]qRT-PCR1 detected only *P. falciparum*.
*Kappa coefficient test for hazard effect: p >0.05.
**Kappa coefficient test for hazard effect: p <0.0001.

microscopy associated with RDT vs. qRT-PCR1, misclassifications were observed for both techniques.

Discussion

The aim of this prospective study was to evaluate three real-time PCR methods in routine diagnosis of imported malaria. Excellent technical results on Ct (inter-assay threshold cycle analysis) and reproducibility of species-related Tms demonstrated the very good reliability of the three qRT-PCR methods for detection and identification of *Plasmodium* sp. Compared with routine methods, qRT-PCR23 both detected all *Plasmodium* sp. infections, whatever the species involved.

The qRT-PCR2 method gave false negatives for three *P. falciparum* infections, routinely diagnosed from the presence of HRP2 antigens only. Compared with thin blood smear examination, the three qRT-PCR methods were more sensitive. These data are similar to other studies, whatever the PCR method used [5] (Table 1). In 17 studies using real-time PCR methods [9–25] (Table 1), a total of 4963 samples were tested. In 1394 samples positive by real-time PCR, optical microscopy detected parasites in only 1317 samples (94.4%) [9–11,13–15,17,19–25]. In our study, blood smear examination and RDT combined allowed detection of all the *P. falciparum* infections. For *P. ovale* or *P. vivax* infection, blood smear examination was more reliable than RDT. We note that in one case, using qRT-PCR allowed diagnosis of a *P. ovale* infection 3 days sooner than conventional tests.

In nine studies, comparing classic PCR to qRT-PCR [10–14,17,20–22] (903 and 898 positive samples, respectively), qRT-PCR was not more sensitive than classic PCR including nested PCR. Regarding species identification, discordance between microscopy and molecular identification has often been reported in the literature, especially for non-*P. falciparum* [11,13,14,21,23,24,26], with PCR used to correct microscopy errors. In our study, no discordance was observed, all the three molecular methods were fully concordant with microscopy and no mixed infection was detected.

Another aspect that has to be taken into account when analysing PCR results is the study conditions. Indeed, in our study it is noteworthy that all patients were clinically suspected of malaria, with indications of fever, and were returning from malaria-endemic countries. These characteristics differ from those of populations studied in endemic areas, where patients may be asymptomatic [20–23,27]; also differing were the PCR methods used for epidemiological data or for screening blood donors [28,29]. In the case of individuals with low-parasitization, PCR interpretation may include the clinical context to determine whether a treatment is needed.

Finally, it is now generally admitted that molecular methods offer excellent specificity and sensitivity and could be considered as a reference standard for malaria infection diagnosis. For routine use, the qRT-PCR method presents advantages of rapidity, less contamination and better standardization. Nevertheless, the qRT-PCR method requires specific material and is more expensive than microscopy [6]. In our hospital laboratories, located in a non-endemic area, our routine methods remain RDT and thin blood smear examination (thick blood smear has been added since 2007). Real-time PCR tests are carried out only in cases where species identification is problematic or for patients strongly suspected of having malaria but with negative results from routine methods.

Conclusion

The three rapid qRT-PCR methods evaluated in this prospective study showed excellent results, used alone or in association. The degree of agreement observed among molecular methods was very high. The three qRT-PCR methods showed accuracy and reliability for malaria diagnosis. In fact, there was no discordance in species identification compared with microscopy. Microscopy associated with RDT should remain a routine diagnostic method. Nevertheless, real-time PCR is the method of choice for low-parasitized individuals, using qRT-PCR1, the most able to detect *P. falciparum* infection, or in cases where species identification is problematic, using qRT-PCR2, which is able to distinguish the four species.

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Transparency Declaration

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References

1. World Health Organization. *World malaria report 2008*. Geneva, Switzerland: WHO Press, 2008.
2. De Pina JJ, Garnotel E, Hance P, Vedy S, Rogier C, Morillon M. Diagnosis of imported malaria in France. *Med Mal Infect* 2007; 37: 710–715.
3. Moody A. Rapid diagnostic tests for malaria parasites. *Clin Microbiol Rev* 2002; 15: 66–78.
4. De Monbrison F, Gerome P, Chaulet JF, Wallon M, Picot S, Peyron F. Comparative diagnostic performance of two commercial rapid tests for malaria in a non-endemic area. *Eur J Clin Microbiol Infect Dis* 2004; 23: 784–786.
5. Berry A, Benoit-Vical F, Fabre R, Cassaing S, Magnaval JF. PCR-based methods to the diagnosis of imported malaria. *Parasite* 2008; 15: 484–488.
6. Berry A, Fabre R, Benoit-Vical F, Cassaing S, Magnaval JF. Contribution of PCR-based methods to diagnosis and management of imported malaria. *Med Trop* 2005; 65: 176–183.
7. Hawkes M, Kain KC. Advances in malaria diagnosis. *Expert Rev Anti Infect Ther* 2007; 5: 485–495.
8. Hermsen CC, Telgt DS, Linders EH *et al.* Detection of *Plasmodium falciparum* malaria parasites *in vivo* by real-time quantitative PCR. *Mol Biochem Parasitol* 2001; 118: 247–251.
9. Lee MA, Tan CH, Aw LT *et al.* Real-time fluorescence-based PCR for detection of malaria parasites. *J Clin Microbiol* 2002; 40: 4343–4345.
10. Fabre R, Berry A, Morassin B, Magnaval JF. Comparative assessment of conventional PCR with multiplex real-time PCR using SYBR Green I detection for the molecular diagnosis of imported malaria. *Parasitology* 2004; 1: 15–21.
11. Rougemont M, Van Saanen M, Sahli R, Hinrikson HP, Bille J, Jaton K. Detection of four *Plasmodium* species in blood from humans by 18S rRNA gene subunit-based and species-specific real-time PCR assays. *J Clin Microbiol* 2004; 42: 5636–5643.
12. Farcas GA, Zhong KJ, Mazzulli T, Kain KC. Evaluation of the RealArt Malaria LC real-time PCR assay for malaria diagnosis. *J Clin Microbiol* 2004; 42: 636–638.
13. Perandin F, Manca N, Calderaro A *et al.* Development of a real-time PCR assay for detection of *Plasmodium falciparum*, *Plasmodium vivax*, and *Plasmodium ovale* for routine clinical diagnosis. *J Clin Microbiol* 2004; 42: 1214–1219.
14. Elsayed S, Plewes K, Church D, Chow B, Zhang K. Use of molecular beacon probes for real-time PCR detection of *Plasmodium falciparum* and other *Plasmodium* species in peripheral blood specimens. *J Clin Microbiol* 2006; 44: 622–624.
15. Mens PF, Schoone GJ, Kager PA, Schallig HD. Detection and identification of human *Plasmodium* species with real-time quantitative nucleic acid sequence-based amplification. *Malar J* 2006; 5: 80.
16. Bialasiewicz S, Whiley DM, Nissen MD, Sloots TP. Impact of competitive inhibition and sequence variation upon the sensitivity of malaria PCR. *J Clin Microbiol* 2007; 45: 1621–1623.
17. Safeukui I, Millet P, Boucher S *et al.* Evaluation of FRET real-time PCR assay for rapid detection and differentiation of *Plasmodium* species in returning travellers and migrants. *Malar J* 2008; 7: 70.
18. Malhotra I, Dent A, Mungai P, Muchiri E, King CL. Real-time quantitative PCR for determining the burden of *Plasmodium falciparum* parasites during pregnancy and infancy. *J Clin Microbiol* 2005; 43: 3630–3635.
19. Swan H, Sloan L, Muyombwe A *et al.* Evaluation of a real-time polymerase chain reaction assay for the diagnosis of malaria in patients from Thailand. *Am J Trop Med Hyg* 2005; 73: 850–854.
20. Mens P, Spieker N, Omar S, Heijnen M, Schallig H, Kager PA. Is molecular biology the best alternative for diagnosis of malaria to microscopy? A comparison between microscopy, antigen detection and molecular tests in rural Kenya and urban Tanzania. *Trop Med Int Health* 2007; 12: 238–244.
21. Boonma P, Christensen PR, Suwanarusk R, Price RN, Russell B, Lek-Uthai U. Comparison of three molecular methods for the detection and speciation of *Plasmodium vivax* and *Plasmodium falciparum*. *Malar J* 2007; 6: 124.
22. Gama BE, Silva-Pires FdoE, Lopes MN *et al.* Real-time PCR versus conventional PCR for malaria parasite detection in low-grade parasitemia. *Exp Parasitol* 2007; 116: 427–432.
23. Veron V, Simon S, Carme B. Multiplex real-time PCR detection of *P. falciparum*, *P. vivax* and *P. malariae* in human blood samples. *Exp Parasitol* 2009; 121: 346–351.
24. Mangold KA, Manson RU, Koay ES *et al.* Real-time PCR for detection and identification of *Plasmodium* spp. *J Clin Microbiol* 2005; 43: 2435–2440.
25. Vo TK, Bigot P, Gazin P *et al.* Evaluation of a real-time PCR assay for malaria diagnosis in patients from Vietnam and in returned travellers. *Trans R Soc Trop Med Hyg* 2007; 101: 422–428.
26. Johnston SP, Pieniazek NJ, Xayavong MV, Slemenda SB, Wilkins PP, da Silva AJ. PCR as a confirmatory technique for laboratory diagnosis of malaria. *J Clin Microbiol* 2006; 44: 1087–1089.
27. Ebrahimzadeh A, Fouladi B, Fazaeli A. High rate of detection of mixed infections of *Plasmodium vivax* and *Plasmodium falciparum* in South-East of Iran, using nested PCR. *Parasitol Int* 2007; 56: 61–64.
28. Fugikaha E, Fornazari PA, Penhalbel Rde S *et al.* Molecular screening of *Plasmodium* sp. asymptomatic carriers among transfusion centers from Brazilian Amazon region. *Rev Inst Med Trop Sao Paulo* 2007; 49: 1–4.
29. Torres KL, Figueiredo DV, Zalis MG, Daniel-Ribeiro CT, Alecrim W, Ferreira-da-Cruz Mde F. Standardization of a very specific and sensitive single PCR for detection of *Plasmodium vivax* in low parasitized individuals and its usefulness for screening blood donors. *Parasitol Res* 2006; 98: 519–524.